Chapter 6

Molecular phylogenetics in the recognition of fungal species, with a particular focus on the Botryosphaeriaceae

ABSTRACT

DNA-based molecular techniques and molecular phylogenetics in species delineation has revolutionised the taxonomy of fungi. Along with deployment of the phylogenetic species concept and genealogical concordance phylogenetic species recognition (GCPSR), cryptic species and species complexes have been revealed where one taxonomic entity was previously known. The *Gibberella fujikuroi* species complex provides one of best examples of fungal plant pathogens, where numerous cryptic phylogenetic species were recognized in one morphospecies. Likewise, the genus *Botryosphaeria* has been radically revised during the past decade based on molecular evidence and a number of new genera and species have been introduced for taxa that previously resided in this genus. This diverse and cosmopolitan group of fungi includes serious plant pathogens as well as some medically important species. In this review, the molecular approaches that are currently applied to delineate fungal species, in particular in the Botryosphaeriaceae, are considered and their implications for the taxonomy of the Botryosphaeriaceae are discussed.

1.0. Introduction

Defining a "species" is fundamental to studies on speciation, understanding of this process and its underlying mechanisms. It is also essential for practical reasons such as disease control and in the application of quarantine regulations. At least 25 different species concepts have been used to define species in the past (Coyne and Orr 2004). These species concepts are classified as theoretical, such as the Evolutionary Species Concept (ESC) (Mayden 1997), or operational of which the more commonly accepted include the Morphological Species Concept (MSC), the Biological Species Concept (BSC) and the Phylogenetic Species Concept (PSC) (Taylor et al 2000). Operational species concepts classify practical criteria that can be used to delineate species (Mayden 1997, Berlocher 1998, de Queiroz 2007). Taylor et al (2000) introduced the term "species recognition" for the operational approaches e.g. Morphological Species Recognition (MSR), Biological Species Recognition (BSR) and Phylogenetic Species Recognition (PSR), in order to distinguish them from theoretical concepts and to emphasize their use in species delimitation, particularly in fungal species diagnoses.

Changes in operational species concepts and the use of PSC and PSR that have been conceptualised in last few decades (Berlocher 1998), have all been influenced by the development of new molecular tools and their availability for species recognition. The most revolutionary change to have arisen is the direct analyses of DNA sequences that became broadly applied in species delimitation in the late 1980's, with the discovery of the Polymerase Chain Reaction (PCR) (Berlocher 1998). Since then, the number of studies on cryptic speciation has increased dramatically in all fields of biology and for all taxonomic groups of living organisms (Bickford et al 2006). One of the important outcomes of the application of molecular based diagnoses has been the recognition that many previously described taxa incorporate cryptic species, which traditionally applied phenotypic characters have failed to reveal.

The application of DNA-based molecular techniques and molecular phylogenetics in species delineation has revolutionised the taxonomy of fungi. Apart from its influence on higher classification, increasing numbers of studies based on DNA sequence variation and application of PSR reveal an escalating number of cryptic species and species complexes in fungal Kingdom (Taylor et al 2000). Based on the outcomes of these studies, it is expected that most of fungal species described based on morphology, comprise more than one closely related cryptic or sibling species, or species complexes. A lack of distinguishing morphological characters, difficulties to induce sporulation in culture, failure of isolates to

mate under laboratory conditions or the lack of living cultures are the main reasons why these species remained cryptic. The increasing number of recognised cryptic fungal species has also necessitated a new approach to the description of these species, and a need to move towards what is referred to as phylogenetic taxonomy.

The rising numbers of species distinguished based on molecular approaches, and cryptic species in fungi in general, is mirrored in the recognition of species and the resulting taxonomy of the Botryosphaeriaceae. Since 1998, when the DNA sequence data were first applied to distinguish species in this family, at least twenty cryptic species have been identified in species of this group, previously defined based on morphology. Numerous others are currently being described. Recently, three cryptic species were described in this family using DNA sequence data and single nucleotide polymorphisms (SNPs) as defining characters for the first time (Pavlic et al 2009b). In this review we consider these developments specifically in the Botryosphaeriaceae, which in many ways provides a leading example that can equally be applied to other fungal groups.

2.0. The historical development of *Botryosphaeria* taxonomy

The Botryosphaeriaceae (Botryosphaeriales, Ascomycota) is referred to here in the strict sense as referring to taxa that were described in the genus *Botryosphaeria*, or anamorphs of *Botryosphaeria*, before 2006, following the classification system of von Arx (von Arx and Müller 1954). This group comprises more than 2000 species (http://www.indexfungorum.com) that are commonly known as endophytes and latent, stress-associated, opportunistic plant pathogens with cosmopolitan distributions on a variety of angiosperms and gymnosperms (Denman et al 2000, Slippers and Wingfield 2007, de Wet et al 2008). Some of the Botryosphaeriaceae are also medically important fungi that may cause diseases in humans (Tan et al 2008, Woo et al 2008).

The taxonomic history and identification of species of *Botryosphaeria sensu* von Arx (von Arx and Müller 1954) can be split in two periods, which are related to pre- and post-the application of the DNA sequence data. The first period started in 1863 when Cesati and de Notaris established *Botryosphaeria*, with 12 species, including *B. dothidea*, which was later identified as the lectotype of the genus (Barr 1972). Until a decade ago, the taxonomy of this group of fungi was based exclusively on morphology, and this period is characterised by morphological species recognition (MSR) (Taylor et al 2000).

Morphological species recognition in *Botryosphaeria* has been complex in the past for a number of reasons. In the initial stages, morphological species identification was

usually combined with a single host-one species approach, which led to the description of new species based on host association (Cesati and De Notaris 1863, Saccardo 1877, 1882, Grossenbacher and Duggar 1911, Putterill 1919). Many of the early-described species were, however, synonymised in a major revision of the genus by von Arx and Müller (1954) based almost exclusively on teleomorph morphology. The occurrence of more than one species on the same host and simultaneous existence of anamorph and teleomorph structures further complicated species identification. Connections between *Botryosphaeria* species and their anamorphs have also not always been available. For example, at the time when *B. dothidea* was described, its anamorph, *Fusicoccum aesculi* Corda, was known, but there were no connections made between these taxa (Pennycook and Samuels 1985, Crous and Palm 1999, Slippers et al 2004b). Identification of species based exclusively on morphological characters either of their anamorphs or teleomorphs is unreliable given that these phenotypic characters overlap between species and in many cases are not sufficiently informative for species delimitation. Denman et al (2000) provided a detailed overview of the taxonomic history of *Botryosphaeria* during this early taxonomic period.

The use of DNA sequence comparisons for the identification and classification of Botryosphaeriaceae was initiated by the study of Jacobs and Rehner (1998). These authors attempted to define species in *Botryosphaeria* and associated anamorphic fungi, combining morphological characters with nuclear rDNA ITS sequence analyses. In this revision, several anamorph genera were linked to *Botryosphaeria* providing the foundation for further taxonomic studies. A subsequent ITS based phylogenetic re-evaluation of *Botryosphaeria* combined with anamorph morphology, by Denman et al (2000), elucidated two main groups for the *Botryosphaeria* anamorphs. These corresponded to species with hyaline, *Fusicoccum*-like conidia and those with dark *Diplodia*-like conidia. Thus, anamorphs of *Botryosphaeria* that were related to 18 different genera were suggested to be synonymised with either *Fusicoccum* or *Diplodia*. The use of the ITS rDNA sequence data for species identification in *Botryosphaeria sensu lato* has subsequently been widely applied (e.g. Zhou and Stanosz 2001, Alves et al 2004, Barber et al 2005, Gure et al 2005, Phillips et al 2006, Pavlic et al 2007).

A major revision of the taxa included in *Botryosphaeria* followed after the analysis of LSU rDNA sequences data by Crous et al (2006). In that study, species of the Botryosphaeriaceae were assigned to at least 10 lineages, which were related to different genera recognised by anamorph morphology. *Botryosphaeria* was reduced to the two species *B. dothidea* and *B. corticis*, and the remaining taxa were accommodated in "*Botryosphaeria*"

quercuum, *Dothidotthia, Guignardia, Neofusicoccum, Neosyitalidium, Macrophomina, Pseudofusicoccum* and *Saccharata,* while the phylogenetic status of *Diplodia* and *Lasiodiplodia* remained unresolved. Resolving the phylogenetic and taxonomic status of dark-spored teleomorph genera in the Botryosphaeriaceae based on a combined phylogeny of five loci (SSU, ITS, LSU, EF-1α and β-tubulin), Phillips et al (2008) recognised *Diplodia* and *Lasiodiplodia* as separate genera, described new dark-spored genera such as *Barriopsis* and *Spencermartinsia*, and re-instated genera *Neodeightonia, Phaeobotryon, Phaeobotryosphaeria* that were synonymised under *Botryosphaeria* by von Arx and Müller (1954). Furthermore, the genus *Dothidotthia* described by Crous et al (2006) was renamed as *Dothiorella*, while *Dothidotthia* species, previously placed in the Botryosphaeriaceae, were shown to belong to the newly established family Dothidotthiaceae (Pleosporales). Recently, two additional anamorph genera, *Aplosporella* (Damm et al 2008) and *Endomelanconiopsis* (Rojas et al 2008), were described in the Botryosphaeriaceae. All of these studies confirm the significance of molecular phylogenetics not only for species level identification but also as an important tool used to resolve the phylogenetic and taxonomic status in higher-level taxa in the Botryosphaeriaceae.

3.0. Phylogenetic species recognition in the Botryosphaeriaceae

In recent years, a number of new or cryptic species have been recognised in the Botryosphaeriaceae (de Wet et al 2003, Slippers et al 2004b, c, d, Burgess et al 2005, Alves et al 2008, Maleme 2008, Phillips et al 2008, Pavlic 2009a, b). Although phenotypic characters were considered in all of these studies, data obtained using molecular markers and DNA sequences, together with the phylogenetic species concept, were used as a foundation on which to base the identification and delimitation of species.

Single locus approach

The Internal Transcribed Spacer (ITS) region of the rDNA operon has been most commonly used for DNA sequence-based identification of fungi (Hajibabaei et al 2007, Nilsson et al 2008). The first DNA-based study on the Botryosphaeriaceae included the sequence data for the ITS region in combination with conidial characters, culture morphology and growth rate to analyse anamorphs of *Botryosphaeria* and related taxa (Jacobs and Rehner 1998). That study indicated that there was not always consensus between morphospecies and phylogenetic clades. For example, strains of *B. dothidea* (anamorph *Fusicoccum aesculi*)

resided in two ITS clades, one of which also included *B. ribis* strains (Jacobs and Rehner 1998). During the course of the decade following that study, numerous studies were conducted in which ITS sequences were used to re-evaluate the relationships amongst known species in this group as well as to confirm the identity and to describe new species (e.g. Denman et al 2000, Smith et al 2001, Zhou and Stanosz 2001, Denman et al 2003, Alves et al 2004, Pavlic et al 2004, 2007, Barber et al 2005, Gure et al 2005, Phillips 2007, Slippers et al 2007).

Comparisons of ITS sequences alone have not always been sufficient to clarify species boundaries in the Botryosphaeriaceae. For example, where isolates of *N. parvum* and *N. ribis* grouped in the same clade in ITS-based phylogenies, they were treated as a species complex or referred to as a *N. parvum* / *N. ribis* clade (Farr et al 2005, Slippers et al 2005, Pavlic et al 2007). In this case, data from ITS sequences were insufficient to either separate these two species or to determine whether other cryptic species existed within this complex. Such observations suggested strongly that there was a need for the inclusion of additional gene sequences or other molecular tools in order to clarify genetic variation observed.

An example of the strengths and limitations of ITS rDNA sequence data can be found in the studies of Pavlic et al (2004, 2007) on Botryosphaeriaceae on native *Syzygium cordatum* trees in South Africa. Prior to these studies, it was thought that *B. dothidea* occurs on this host (Smith et al 2001), but it was later shown that the isolates from *S. cordatum* represented *N. parvum* (Slippers et al 2004b). ITS rDNA sequence data, combined with anamorph morphology and PCR-RFLP analyses of the same region, later revealed that eight species occur on this host, of which *L. gonubiensis* was described as new (Pavlic et al 2004, 2007). Although the ITS phylogeny was sufficient to discriminate *L. gonubiensis* in these studies, this region alone could not separate the two closely related species *N. parvum* and *N. ribis*. Isolates within the *N. parvum* / *N. ribis* complex exhibited much variation in conidial morphology and ITS sequences. However, support for the sub-clades obtained in phylogenetic analyses of ITS sequence data was very low, leaving uncertainty as to their interpretation.

Multiple locus approach

The limitations of using single locus sequence data, especially for closely related sister species where ITS rDNA do not provide sufficient resolution, has led to sequences for more than one locus being used to delimit species in recent years. Examples can be found in studies on *Neurospora* and *Gelasinospora* (Dettman et al 2001, 2003), the human pathogenic

fungus *Cryptococcus neoformans* (Xu et al 2000), and other important human and plant pathogenic fungal complexes, such as *Fusarium graminearum* and *Gibberella fujikuroi* (O'Donnell et al 2000a, b, Steenkamp et al 2002, O'Donnell et al 2004), *Trichoderma harzianum / Hypocrea lixii* complex (Chaverri et al 2003), *Aspergillus flavus* and *A. fumigatus* (Geiser et al 1998, Pringle et al 2005), *Coccidioides immitis* (Koufopanou et al 1997) and many others. In all of these studies, various previously unidentified, cryptic phylogenetic species were revealed.

Genealogical concordance phylogenetic species recognition (GCPSR) was applied to the gene genealogies of multiple loci in the studies described above, in order to identify cryptic species. The GCPSR is a form of PSR that has most commonly been applied to study members of the fungal Kingdom (Taylor et al 2000). By relying on concordance of more than one gene genealogy, this method eliminates the limits of application of phylogenetic analyses of single genes (Taylor et al 2000).

GCPSR based on multi-locus sequences was first applied in a study on Botryosphaeriaceae by de Wet et al (2003). In that study, partial sequences of six protein-coding genes and six microsatellite loci, were used to elucidate phylogenetic relationships amongst isolates of *Diplodia pinea* (= *Sphaeropsis sapinea*) representing the A, B and C morphotypes previously described for this fungus. Although these morphotypes were described based on differences in pathogenicity, morphological and molecular characters, it was not clear whether they represent different taxa, because some characters overlapped between them. Application of GCPSR provided evidence that the B morphotype isolates were genetically distinct from *D. pinea* and this morphotype was consequently recognised as a new species described as *D. scrobiculata* (de Wet et al 2003). This was the first species in the Botryosphaeriaceae identified by the explicit application of GCPSR.

The application of GCPSR has been used to resolve long-standing uncertainty regarding the existence of cryptic species in the *N. parvum / N. ribis* complex. *Neofusicoccum parvum* and *N. ribis* were described as separate taxonomic entities based on morphological features (Grossenbacher and Duggar, 1911, Pennycook and Samuels, 1985). Although combined sequences for three gene regions separated these species (Slippers et al 2004b), they could not be delineated in many other studies, even where multiple gene sequences were used. This raised the question as to whether cryptic species were present in the complex. In the study of Pavlic et al (2009a), using sequences from five loci and GCPSR, three cryptic species were identified in the *N. parvum / N. ribis* complex from

Syzygium cordatum in South Africa. These species were described as *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* (Pavlic et al 2009b).

Increased numbers of cryptic species have been detected in different genera of the Botryosphaeriaceae using multiple gene phylogenies generated using ITS rDNA, EF-1α and β-tubulin sequence data. Phylogenetic analyses distinguished *N. eucalypticola* from *N. eucalyptorum* (Slippers et al 2004c) and *N. australe* as a sister species to *N. luteum* (Slippers et al 2004d). The same method was recently used to separate of *N. crypto-australe prov. nom.* as an additional sister species in the *N. luteum* / *N. australe* complex (Maleme 2008). Another example of species delineation using multiple gene sequences can be found in the morphologically similar species *B. dothidea* and *N. ribis* (= *B. ribis*) that were thought to represent a species complex. In a study by Zhou and Stanosz (2001), the ITS phylogeny supported separation of these two species that could not clearly be distinguished in the study of Jacobs and Rehner (1998). Using combined multiple gene sequences of the ITS rDNA, EF-1α and β-tubulin gene regions, along with phenotypic characters, Slippers et al (2004a) clarified the identity of *B. dothidea* and *N. ribis,* as well as *N. parvum* (= *B. parva*). In all of these studies, genetic variation observed within the clades in the phylogenetic analyses based on ITS sequences alone, gave a clear indication of new species, although their identity could only be clarified using multiple gene sequences.

Based on combined ITS and EF-1α sequences, a number of new species have been recently been recognised in *Diplodia, Lasiodiplodia* and *Dothiorella* (Luque et al 2005, Phillips et al 2005, Burgess et al 2006a, Damm et al 2007, Lazzizera et al 2008). For example, this approach was used to identify *Diplodia cupressi,* previously known as *D. pinea* f. sp. *cupressi*, as a distinct species (Alves et al 2006). It was also used to identify cryptic species, *L. pseudotheobromae* and *L. parva,* among a collection of isolates previously identified as *L. theobromae* (Alves et al 2008). Although not always explicitly applied as the phylogenetic species concept or phylogenetic species recognition, but rather as combined phylogenies used to clarify the identity of unresolved taxa based on single gene phylogeny, these studies represented the first steps towards PSR.

Microsatellite marker data

Single Sequence Repeat (SSR) or microsatellites are short repeat sequences found throughout the genomes of Eukarya that are commonly used as co-dominant markers in various typing studies. Amplified loci that contain SSR repeats can be analysed for variation in sequence data or for fragment size variation that depends on the number of repeats

contained in the microsatellite region (Squirrell et al 2003). This method can be used in combination with multilocus gene sequences as a form of multilocus species typing (MLST), typically used in studies of bacterial diversity (Taylor and Fisher 2003). Such microsatellite markers were, for example, used in the diagnosis of the phylogenetically recognised human fungal pathogens *Coccidioides posadassi* as well as in cryptic species in *Paracoccidioides brasiliensis* (Fisher et al 2002, Matute et al 2006). These studies showed that microsatellite loci could be used as molecular markers to characterise and type strains, as well as to assign strains to the described species. They could thus provide a simple and reliable means for the identification of genetically recognised cryptic species.

Microsatellites have been used for typing of populations and cryptic species in the Botryosphaeriaceae, especially in the pine pathogen *D. pinea* and related species (Burgess et al 2002, de Wet et al 2003). Microsatellite markers designed for this fungus clearly distinguished the three morphotypes of *D. pinea* (de Wet et al 2000, Burgess et al 2001, de Wet et al 2002). The sequences of the microsatellite regions were also used in combination with sequences from introns of six functional genes to analyse the relationship between morphotypes of *D. pinea*, and to distinguish *D. scrobiculata* amongst them (as discussed above) (de Wet et al 2003). Comparison of the multiple gene genealogies in the latter paper with those from the sequenced microsatellite loci confirmed that the sequences of microsatellite markers alone would be adequate for species recognition.

Microsatellite markers have been developed for *N. parvum*, but these also amplify corresponding loci in a few other Botryosphaeriaceae with *Fusicoccum* and *Neofusicoccum* anamorphs (Slippers et al 2004a). They have further been used in a population study on *N. australe* to show gene flow between native forests and plantations of *Eucalyptus globulus* in Western Australia (Burgess et al 2006b). These microsatellite markers have also been useful to delineate cryptic species in the *N. parvum* / *N. ribis* complex, and appropriate for analyses of inter- and intra-specific variation and population structure of sister species *N. cordaticola*, *N. kwambonambiense, N. umdonicola* and *N. parvum* (Pavlic et al 2009c).

Other molecular tools

The application of multiple gene genealogies and SSR markers is critical for the identification of cryptic species in the Botryosphaeriaceae. However, these methods can be time consuming and expensive and there is a need for accurate and rapid screening protocols following the initial delineation of the species. One approach that can be used is to find characteristic SNP or SSR alleles that characterize a species. Such data have been used to

develop PCR-RFLP fingerprinting techniques to distinguish species in the Botryosphaeriaceae. For example, the *sensu lato* groups of *N. parvum* and *N. ribis* could be distinguished based on *Cfo*I digestion of an SSR locus (*BotF15*) (Slippers 2003). Similarly, Alves et al (2005) used amplified ribosomal DNA restriction analyses (ARDRA) to differentiate isolates of twelve Botryosphaeriaceae species. Recently, Alves et al (2007) designed MSP-PCR (microsatellite-primed polymerase chain reaction) and rep-PCR (repetitive-sequence-based polymerase chain reaction) fingerprinting methodologies for the rapid identification of Botryosphaeriaceae species, including closely related species such as *N. parvum* and *N. ribis*, or *N. luteum* and *N. australe*. All of these techniques provide rapid and simple methods that can be readily used in species identification in the Botryosphaeriaceae. Thus, further application of such tools should be considered for newly identified phylogenetic species.

4.0. Towards phylogenetic systematics in the Botryosphaeriaceae

The majority of the more than 70000 described fungal species (Hawksworth et al 2004) have been defined based on morphological or other phenotypic characters, also referred to as MSR. However, speciation is not always correlated with morphological change (Taylor et al 2000). Comparisons of MSR and PSR have, therefore, not surprisingly shown that PSR performs the best, because changes in gene sequences occur and can be diagnosed before changes have occurred in mating behavior or morphology (Taylor et al 2000). Biological Species Recognition (BSR), which is commonly used in other fungi such as, for example, in the *Gibberella fujikuroi* complex (Leslie 1995, Kvas et al 2009), has not been applied to the Botryosphaeriaceae, because they do not produce sexual structures in culture. The BSR is, therefore, not be considered further here. Thus, with the application of PSR, numerous species have been identified that were previously morphologically or biologically cryptic, due to the lack of taxonomically informative phenotypic characters or incomplete reproductive isolation amongst the species. This reality is driving a need for changes to the way that the taxonomy of fungi is approached, and this is especially true for the Botryosphaeriaceae.

Due to their plasticity, inconsistency and overlapping nature, morphological features have been insufficient to distinguish closely related or sister species of the Botryosphaeriaceae with confidence. However, in many studies on the Botryosphaeriaceae, preliminary groupings of isolates have been based on cultural and conidial morphology (e.g. Slippers et al 2004a, Burgess et al 2005, Pavlic et al 2007, 2008). In these studies, groups

identified based on morphological characters were usually found congruent with those recognized based on DNA sequence data and *vice versa*. Although some morphological characters commonly used in the identification of Botryosphaeriaceae, such as conidial and ascospore shape, size, septation, wall thickness and color, as well as culture morphology and pigmentation, have provided strong indication of potentially cryptic species, further confirmation using other less subjective tools has typically been required.

Culture morphology has been useful to distinguish between some species in the Botryosphaeriaceae. For example, *N. luteum* was distinguished from related species by a yellow pigment formed in young cultures (Pennycook and Samuels 1985). However, some of isolates included in a study by Slippers et al (2004d), that were originally thought to represent this species based on conidial and culture morphology, exhibited slight differences in pigmentation from the original strains of *N. luteum*. However, differences in ITS rDNA sequence data amongst *N. luteum* isolates in that and other studies (Smith and Stanosz 2001, Denman et al 2003) were inordinately small to make conclusive decisions regarding potential cryptic species. It was only after fixed alleles across multiple gene regions indicated a genetic barrier between the groups representing the different cultural morphologies that *N. australe* could be described as distinct (Slippers et al 2004d). Culture morphology has also been useful in separation of other species in the Botryosphaeriaceae, but in many cases molecular support remained necessary to confirm species boundaries (de Wet et al 2003, Burgess et al 2005, Pavlic et al 2008).

Conidial morphology has been most extensively used in species identification in the Botryosphaeriaceae. It has been shown that variation in conidial morphology can indicate species diversity, but could not *a priori* confirm species differences. For example, isolates that resided in the *N. parvum* / *N. ribis* complex from *S. cordatum* in South Africa exhibited high levels of variation in conidial measurements and morphology, which differed from those in the original descriptions of *N. parvum* and *N. ribis*. This suggested that additional species could exist in this complex (Pavlic et al 2007). In a follow-up study (Pavlic et al 2009a), the selection of isolates for DNA sequencing based on variation in conidial mophology proved to be useful to sample representatives of different cryptic species, which were then recognised as *Neofusicoccum* spp. R1, R2 and R3 in that paper. When additional isolates were identified using molecular tools and included in morphometric analyses, a continuum in conidial variation was observed for these phylogenetically recognised species (Pavlic et al 2009b). Thus, while morphological differences can be used for initial selection

of isolates from a larger collection prior to molecular identification, species can not be identified based on this character alone.

The discovery of morphologically cryptic species using molecular tools makes it technically impossible to describe them taxonomically, because the International Code of Botanical Nomenclature requires morphologically distinct characters. This has led to the description of species using molecular characters (DNA sequence data), although this is not strictly allowed by the Code. The phylogenetic species are characterized primarily by fixed single nucleotide polymorphisms (SNPs) (O'Donnell et al 2004, Grünig et al 2008, Pavlic et al 2009a, b). Although many cryptic, phylogenetic species have been recently recognized in the fungal Kingdom, there are very few descriptions of these species. Some examples include the description of human pathogen *Coccidioides posadasii* (Fisher et al 2000), nine phylogenetically distinct species within *Fusarium graminearum* clade (O'Donnell et al 2004) and six cryptic species of the *Phialocephala fortinii* s.l.-*Acephala applanata* species complex (Grünig et al 2008). Fixed nucleotide characters, given by gene and nucleotide position were used in diagnoses of these species. Without the descriptions, these phylogenetically recognised species would remain cryptic. Increasing numbers of phylogenetic species that cannot be diagnosed based on morphological characters, provide a strong case for changes to the regulations regarding descriptions of fungal species.

The first phylogenetically recognized species in the Botryosphaeriaceae were recently described using sequence data and SNPs as defining characters (Pavlic et al 2009b). Three new species were thus recognized in the *N. parvum* / *N. ribis* complex as *Neofusicoccum* spp. R1, R2 and R3 using multiple gene genealogies and GCPSR (Pavlic et al 2009a) (Fig. 1) and described as *N. cordaticola, N. kwambonambiense* and *N. umdonicola*, respectively (Pavlic et al 2009b). The conidial sizes of these species lie in a continuum, and can not be used to distinguish the species *a priori* the application of DNA sequence data (Fig. 2). Analyses of conidial measurements following molecular identification, however, revealed statistically significant differences between the average conidial dimensions for *N. parvum*, *N. cordaticola*, *N. kwambonambiense* and *N. umdonicola* (Pavlic et al 2009b). Even with this information, their correct diagnosis can only be achieved by SNPs (Pavlic et al 2009b). Phenotypic characters can identify many cryptic species once revealed using molecular tools, however due to small differences in morphological features, these species must be described using molecular characters.

5.0. Utility of one name per fungal species

The International Code of Botanical Nomenclature (ICBN) requires two separate names for the anamorph and teleomorph states of fungi (Article 59) (McNeill et al 2005). Where both are known, the teleomorph name takes preference. The growing numbers of fungal species that have been identified based on DNA sequence data or using other molecular tools are, however, intensifying the need to use one name per fungal species. This is because new species can be linked to either teleomorph or anamorph genera based on DNA sequence data, irrespective of what state of the fungus was collected or could be induced in culture. Furthermore, many morphologically defined teleomorph genera are being shown to include numerous anamorph genera, and some anamorph genera are clearly polyphyletic (Crous et al 2006, Phillips et al 2008). Application of molecular tools allows us to define relationships of asexually producing fungi and establishes anamorph-teleomorph connections based on molecular phylogeny, even when the teleomorph state is unknown. Consequently the holomorph concept that provides one name per fungal species that reflects the phylogeny of taxa (Rossman and Samuels 2005) should be more widely accepted by the mycological community. Although a proposal for a single scientific name for fungi was recently made (Rossman and Samuels 2005), it appears that the transition towards the application of one name for a fungal taxon will not be an easy task.

A one species-one name approach to fungal taxonomy would aid the taxonomy of the Botryosphaeriaceae, since the sexual state is less commonly found in the nature than the anamorph and it is also rarely induced in culture. This problem was highlighted in a recent study when phylogenetic lineages in the Botryosphaeriaceae (all previously linked to the teleomorph *Botryosphaeria*) were characterized as distinct genera based on anamorph differences (Crous et al 2006). Not all these genera could be linked to teleomorph taxa, and many of the genera recognised by Crous et al (2006) are, therefore, known only by their anamorph names. An example is found in the well-known pathogen of fruit and fruit trees, '*Botryosphaeria*' *obtusa*. Following the taxonomic changes this name was no longer valid and only the anamorph name, *Diplodia seriata*, can be used (Phillips et al 2007). Similarly, *Lasiodiplodia theobromae* now represents '*Botryosphaeria*' *rhodina*, *Diplodia mutila* represents '*Botryosphaeria*' *stevensii*, *Neofusicoccum parvum* represents '*Botryosphaeria*' *parva* and many more (Crous et al 2006). These changes reflect the evolutionary divergence amongst the groups much more accurately than was true in previous taxonomic treatments. They also facilitate thinking and communication regarding the evolutionary history of the groups (de Wet et al 2008).

6.0. DNA-barcoding

The DNA Barcode of Life Initiative aims to provide unique DNA sequences, the 'barcode,' for the identification of all biological species (http://barcoding.si.edu/). The ITS rDNA locus is currently the preferred region to serve as the universal tool in identification of fungal species (Nilsson et al 2006, 2008; www.allfungi.org/its-barcode.php). Although ITS rDNA sequences have been broadly used for fungal DNA based identification (Hajibabaei et al 2007, Nilsson et al 2008), it has been argued above that this region alone is not sufficient to distinguish closely related or cryptic species of the Botryosphaeriaceae (e.g. Smith et al 2001, Slippers et al 2004b, Farr et al 2005, Pavlic et al 2007). This is also true for many species in other groups of fungi (Bruns and Shefferson 2004, Bischoff et al 2006, Kvas et al 2009). However, no other region currently provides a more suitable basis for barcoding the Botryosphaeriaceae in terms of ease of amplification and distinguishing power. Despite its shortcomings, the ITS rDNA thus remains the most suitable region to serve as an effective barcoding locus.

The fact that the majority of fungal species are described based on morphology alone, presents a significant challenge to DNA barcoding efforts. This challenge is also substantial in the Botryosphaeriaceae. Although more than 2000 species are known in the Botryosphaeriaceae (www. indexfungorum.org), a limited number are represented by sequence data in GenBank or even cultures. The application of DNA sequence based characterisation without consulting previous descriptions based on phenotypic characters can thus lead to the description of species that have already been described, but for which sequence data are not available. Furthermore, not all the sequences in GenBank are linked to type specimens and might not represent the taxa they are labelled with. In general, as many as 27 % of ITS rDNA fungal sequences deposited in GenBank have been found to be from wrongly identified specimens and cultures (Nilsson et al 2006). Much work thus remains to be done, before a reliable database will exist and upon which DNA based taxonomy and DNA barcoding systems can rely.

7.0. Consequences of phylogenetic species recognition in the Botryosphaeriaceae

Similarly to other fungal groups (see Le Gac et al 2007), the application of molecular tools has contributed substantially to our understanding of host relationships in the Botryosphaeriaceae. Some species previously thought to be generalists have been shown to represent complexes of species and cryptic species, which are specialists on one host or on a

few related hosts. For example, *B. dothidea* was considered to be a widely distributed species on a variety of native and cultivated hosts. Species previously treated under *B. dothidea* are now known to vary from specialists such as *N. eucalyptorum* on *Eucalyptus* and *N. protearum* on Proteaceae, to generalists such as *N. parvum* that has been associated with a variety of hosts worldwide (Slippers and Wingfield 2007, de Wet et al 2008). It is clear that incorrect species identifications underestimate the diversity of fungal communities on host plants and they also obscure the specificity of many species.

Accurate species identification is important for understanding patterns in the distribution of the Botryosphaeriaceae and to implement suitable quarantine measures to reduce the probability of their spread to new environments. Species of the Botryosphaeriaceae are known as endophytes that can easily be moved unnoticed into new areas (Burgess and Wingfield 2002, Slippers and Wingfield 2007). Once introduced into new areas, they are likely to infect new hosts. For example, a recent population study on the plant pathogen *L. theobromae* revealed high gene flow between populations from three different hosts in Venezuela, *Pinus caribaea*, *Eucalyptus urophylla* and *Acacia mangium,* and indicated that there was no host specificity for isolates of this fungus (Mohali et al 2005). Movement of these species between native and non-native hosts and their introduction into new areas could pose a serious threat to agricultural crops, trees in plantations and native flora.

Recently, eight species of the Botryosphaeriaceae were identified from native *S. cordatum* in South Africa (Pavlic et al 2007). These species were also shown to be able to infect *Eucalyptus* and were more virulent on this host than on *S. cordatum*, at significantly different levels (Pavlic et al 2007). Isolates treated as *N. ribis*-like in the study of Pavlic et al (2007), were later shown to represent three cryptic species that were significantly more virulent than *N. parvum* and *N. ribis* to *S. cordatum* in greenhouse trials (Pavlic et al 2009b). Isolates identified as '*N. ribis*' were also highly pathogenic to different *Eucalyptus* clones grown commercially in Venezuela (Mohali et al 2009) and Colombia (Rodas et al 2009), but the identity of these isolates remains to be confirmed. Other examples include the *Diplodia pinea* morphotypes and *D. scrobiculata* that differed in virulence to *Pinus* (de Wet et al 2000, 2003). Inoculation trials on different host plants, such as *Eucalyptus* and grapevines identified *B. dothidea* as least virulent, while *N. parvum* was amongst the most virulent Botryosphaeriaceae tested by van Niekerk et al (2004) and Pavlic et al (2007). Two closely related species in *Lasiodiplodia, L. theobromae* and *L. gonubiensis,* differ significantly in their virulence, with *L. theobromae* being more virulent (Pavlic et al 2007). Since isolates of

cryptic species differ in virulence, their correct identification is of enormous importance for control purposes and management strategies. Application of the phylogenetic species concept will allow us to recognise morphologically and ecologically cryptic species in under-explored environments, such as natural stands of different species of plants. This has been particularly evident in the Botryosphaeriaceae where applications of DNA based tools in species identification have revealed substantial unknown diversity in recent years (Pavlic et al 2004, Slippers et al 2005, Pavlic et al 2008, van der Walt 2008, Taylor et al 2009).

In two extensive studies recently conducted on more than thirty native tree species, including *Adansonia digitata* (baobab), *Acacia* spp. and *Eucalyptus gomphocephala,* eleven new species of Botryosphaeriaceae were described (Pavlic et al 2008, Taylor et al 2009). An additional twelve new species were recognised from native *Acacia* spp. in Southern Africa (van der Walt 2008). Discovery of many new fungal species on the plants in native environments indicates that plants in natural stands are under explored and will most likely harbour numerous new species. These findings underpin the necessity of having a holistic view of fungal communities on native and planted trees in order to record and conserve their true diversity.

Molecular tools have proven useful in the identification of medically important species in the Botryosphaeriaceae (Tan et al 2008, Woo et al 2008). The fungi identified in these studies included *L. theobromae, Macrophomina phaseolina* and *Neoscytalidium dimidiatum* (= *Scytalidium dimidiatum*). Interestingly, all of these species are also well-known plant pathogens (Punithalingam 1976, Crous et al 2006, Avilés et al 2008). It is thought that in all of the cases, humans were infected through environmental exposure and through contact with contaminated plant material and soil (Tan et al 2008). What triggers these species to infect and cause diseases in humans will need further clarification. However, identification of these clinical isolates based on morphology was difficult. For example, one of the isolates was initially thought to represent, *Pseudallescheria boydii*, based on colony morphology, and then later identified as *L. theobromae*, of which identity was also uncertain since the isolate failed to produce fruiting structures. The ITS rDNA sequence comparisons, however, determined the isolate as *Macrophomina phaseolina* (Tan et al 2008). This is another example that highlights the necessity of using molecular tools in the correct identification of species in Botryosphaeriaceae, which can be particularly difficult in non-sporulating isolates.

8.0. Conclusions

Phylogenetic inference based on DNA sequence data has had an enormous impact on the taxonomy of the Botryosphaeriaceae. At the species level, a phylogenetic approach has revealed that a number of previously well defined taxa encompass cryptic species that had previously been overlooked. The result has been the description of numerous new species, many of which can hardly be distinguished from their sister species based on morphology. DNA sequences have also been used in the re-evaluation of *Botryosphaeria sensu lato* and its placement in higher orders of fungal classification. Thus, new genera have been recognised and their phylogenetic relationships, anomorph-teleomorph connections and placement in the family have been clarified.

Although ITS rDNA sequence comparisons were useful at the early stages of DNA based identification of the Botryosphaeriaceae, and fungi in general, sequences for additional loci often revealed cryptic species that could not be delineated based on ITS rDNA sequences alone. Through these studies GCPSR, as a form of PSR based on concordance of multiple gene genealogies, has emerged as the most powerful tool in species recognition. It is expected that through the application of this approach, new species and species complexes will be discovered. Although it is debatable whether ITS rDNA region will be most suitable for DNA barcoding in fungi, molecular phylogenetics will provide the most important basis for species identification as well as for molecular systematics.

The application of molecular tools other than single or multiple locus sequence data, such as microsatellite markers, and a polyphasic approach will lead to more detailed insights into inter- and intra-species diversity for the Botryosphaeriace. This will improve our knowledge of evolution of fungal species and understanding of processes that drive speciation. It will further advance and clarify criteria for species delineation and assist in the identification of species boundaries among closely related species and species complexes. It is, however, apparent that this is a process that is far from complete and many new species of agricultural or medical importance have yet to be discovered.

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FIG. 1. One of two unrooted maximum-parsimony trees resulting from the analysis of the combined sequence data of five loci, including ITS rDNA, EF-1α, RPB2, the Bt2 region of the β-tubulin gene and *BotF15*, shows distinct clades for *N. parvum, N. ribis, N. cordaticola, N. kwambonambiense* and *N. umdonicola*. The combined sequence data analysis, and in particular also the linked divergence indicated by the individual gene genealogies (data not shown), indicate species barriers that was not evident by considering morphological characters alone. Bootstrap values of maximum parsimony analyses are indicated next to the branches followed by the posterior probabilities resulting from Bayesian analysis (indicated in italics). Isolates obtained from *S. cordatum* are indicated in bold. Ex-type isolates and isolates linked morphologically and geographically to the types of *N. parvum* and *N. ribis* are underlined. Isolate numbers are those of the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, South Africa.

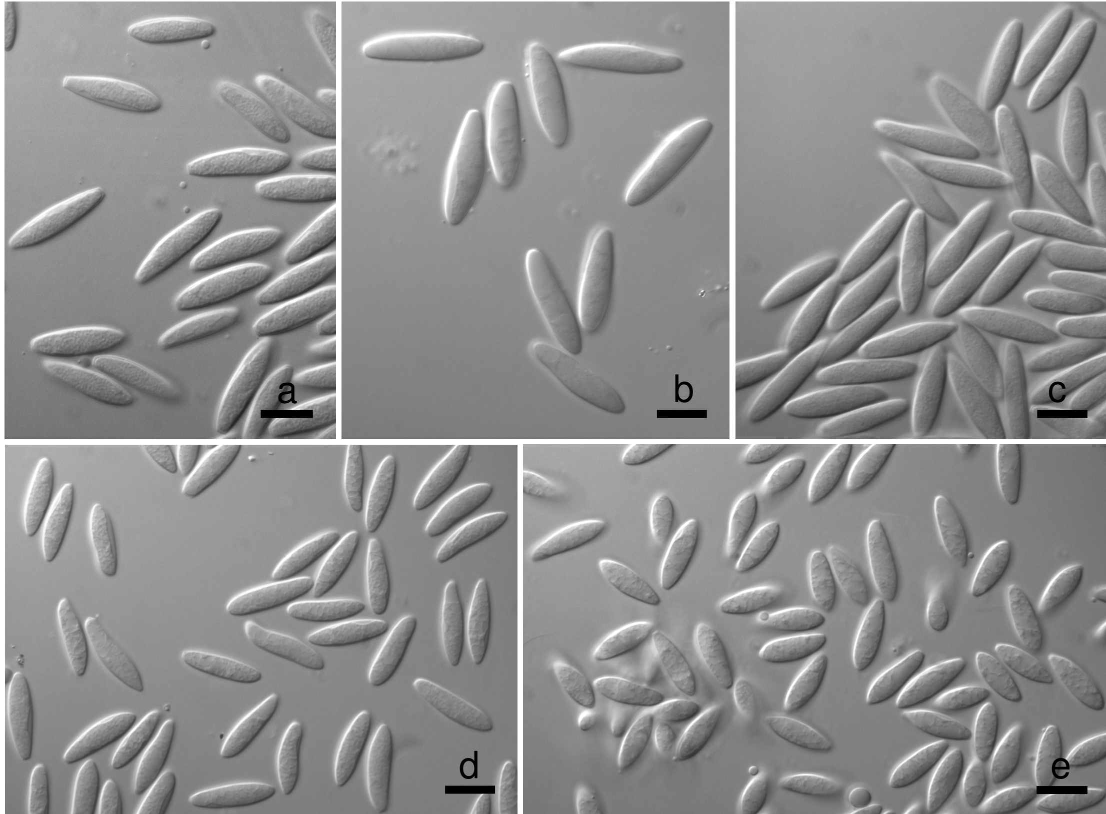
a
b
c
d
e

FIG. 2. Conidia of four cryptic species in the *N. parvum* / *N. ribis* complex recognised as *N. umdonicola* (a), *N. kwambonambiense* (b), *N. cordaticola* (c) and *N. parvum* (d, e) using GCPSR of five sequenced loci. These species cannot be distinguished from each other with certainty based on conidial morphology alone, which was commonly used in the past for this purpose.

14085
14094
64/*1.0*
14088
14135
14141
27901
14097
14080
14087
14082
14143
87/*1.0*
14029
100/*1.0*
9080
63/*1.0*
14089
65/*1.0*
9079
95/*1.0*
70/*0.97*
9081
14129
14155
99/*1.0*
62/1.00
14140
100/*1.0*
14023
52/*0.91*
14025
13992
100/*1.0*
14124
64/*0.99*
14123
100/*1.0*
14151
99/*1.0*
7772
14054
14056
99/*1.0*
7773
14127
63/*1.0*
7054
14079
14096
14060
14058
14106

Neofusicoccum cordaticola
N. kwambonambiense
N. umdonicola
N. parvum
N. ribis